LightMix® *in-vitro diagnostics kit*
HFE H63D S65C C282Y

Cat. -No.: 40-0340-32
Roche SAP No.: 05945798001

Detection of the HFE gene DNA variations
encoding for p.H63D, p.S65C and p.C282Y

for use with the

Roche Diagnostics LightCycler® Instruments

Reagents for 96 reactions

Upon arrival:

Store dried Premixed PCR reagents and Controls protected from light at room temperature (do not freeze)

CE IVD

Table on Contents

1. Product Information

Dried premixed PCR reagents

⚠ **Store at 4 °C to 25 °C protected from light**

	Cap color	Label	Description content	Reaction / Tube status	Total
3 x	**Red**	**PSR**	Parameter-Specific Reagent (PSR) contains premixed and dried primers and probes for 32 reactions each. ≤ 86 % Synthetic oligonucleotides ≥ 14 % Buffer	32 reactions green-blue pellet dried	96 rxs

Standards (Control DNA)

⚠ **Store at 4 °C to 25 °C protected from light**

	Cap color	Label	Description content	Reaction Tube status	Total
1 x	**Yellow**	**Standard 1**	Positive Control HFE Mutant 282Y and wild type for all other positions about 4E5 genome equivalents	32 reactions blue pellet dried	32 rxs
1 x	**Yellow**	**Standard 2**	Positive Control HFE Mutant 63D and wild type for all other positions about 4E5 genome equivalents	32 reactions blue pellet dried	32 rxs
1 x	**Yellow**	**Standard 3**	Positive Control HFE Mutant 65C and wild type for all other positions about 4E5 genome equivalents	32 reactions blue pellet dried	32 rxs

1.2 Intended Use

This kit allows to detect common mutations in the HFE gene (OMIM: 235200) in genomic human DNA from nucleic acid extracts obtained from peripheral blood. *Hemochromatosis* type 1 (HFE1) is hereditary and linked to various mutations in the HFE gene. Hemochromatosis results in multi-organ dysfunction caused by increased iron deposition.

This product is intended to help clinicians to analyze the genetic background of patients with hepatopathy of unknown etiology, patients with liver cirrhosis, diabetes mellitus, bronze skin pigmentation in connection with elevated serum iron concentrations, elevated transferrin saturation and elevated serum ferritin levels.

The present test can be performed in addition or after a biochemical assay for iron overload using transferrin saturation.

Results obtained using this kit are not intended to be the only basis for any therapy decision. The patient´s mutation status should be considered alongside other disease factors.

Note: The performance of the assay can be guaranteed only when used with Roche LightCycler® Instruments or cobas z 480 Analyzer (see 1.3.2 for details).

The present product is an *in-vitro* diagnostic device, which must be used by qualified personnel only.

1.3 Specifications

The LightMix® Kit *HFE H63D S65C C282Y* is an *in-vitro* diagnostic test, which allows to detect and identify the clinical relevant single nucleotide polymorphism variants p.H63D, p.S65C and p.C282Y in the HFE gene.

The presence of other gene variants may interfere with the test. The following exon 4 rare variants will be also detected (see 3.1) but not 'identified' :

p.T281M	c.842C>T	rs915273578	≈ ΔTm -15 °C
p.T281T	c.843G>A	rs369354634	
p.Q283P	c.848A>C	rs111033563	≈ ΔTm - 8 °C

1.3.1 Clinical Samples

The test requires 5 µl of purified genomic DNA in an aqueous solution extracted from clinical specimen (peripheral blood), containing 3 to 100 ng/µl of genomic DNA (15 – 500 ng total amount), with the DNA concentration determined by UV spectrophotometry (1 OD_{260} = 50 µg DNA/ml).

1.3.2 Instruments, Software and Productivity

One kit contains reagents for 96 reactions performed in a 20 µl volume.
Each run requires including three standards and one No Template control.
The table below summarizes some features of the kit :

LightCycler® Instrument	Software Version (or higher)	Run Time (approx.)	Max Samples per run (2)	Maximum Productivity of the kit (3)	Minimum Productivity of the kit (4)
LC 1.2	4.10 (1)	60 min	28 + 4 ctrl.	81	18
LC 1.5	4.10 (1)	60 min	28 + 4 ctrl.	81	18
LC 2.0	4.05	60 min	28 + 4 ctrl.	81	18
LC480 (96 wells)	1.5	100 min	92 + 4 ctrl.	89	18
LC480 (384 wells)	1.5	100 min	380(5) + 4 ctrl.	89	18
z 480 (open channel)	1.5	100 min	92 + 4 ctrl.	89	18
LC PRO	1.X.X	70 min	89(6) + 7 ctrl.	89	12

1 Running the test with the LightCycler® 1.2 or 1.5 Instruments with the software version 3.5 yields comparable results. Instructions for software 3.5 programming, data analysis and interpretation of results are not described in this manual. Upgrade to version 4.10 or higher when possible.
LightCycler® software 3.5.3 does not contain the automatic genotyping module; equivalent results can be obtained by trained personnel which must analyze each sample manually.
2 Each run must include 3 standards and one No Template Control (NTC) for a total of 4 control reactions.
3 The first run of the kit requires including 7 controls (instead of 4) to teach the genotyping module. The maximum number of samples that can be processed is reduced accordingly.
Depending on local regulations, all 7 genotyping controls may have to be included in each run, reducing the total number of patient's samples that can be analyzed.
4 Calculated considering a single clinical sample analyzed in each run.
5 It requires using four kits.
6 For LightCycler® PRO each run must include all three genotyping standards (1-3), the combinations of standards (1+2, 1+3 & 2+3) and one NTC.

1.4 Storage and Stability

Storage Conditions

Reagents and Controls

Store the dried reagents (PSR and Standards) protected from light and at room temperature or refrigerated (from 4 °C - 25 °C).
Do not freeze dried reagents. Expiration date is printed on the kit label.

2. Additional Devices and Reagents

2.1 Color Compensation

	TIB Molbiol
LightMix® Kit – Color Compensation HybProbe (not required for LightCycler® PRO)	Cat.-No. 40-0318-00

2.2 LightCycler® Instruments

LightCycler® 2.0 Instrument	**Roche Diagnostics**
LightCycler® 2.0 Instrument	Discontinued
LightCycler® Software Version 4.05 or	Discontinued
LightCycler® Software Version 4.10 or higher	Cat.-No. 04 898 915 001
LightCycler® Capillaries (20 µl)	Discontinued
Or	
LightCycler® 480 Instruments	**Roche Diagnostics**
LightCycler® 480 Instrument (model I)	Discontinued
LightCycler® 480 II Instrument	Cat.-No. 05 015 278 001
cobas z 480 Analyzer	Cat.-No. 05 200 881 001
LightCycler® Software Version 1.5 or higher	Cat.-No. 04 994 884 001
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
Or	
LightCycler® 1.x Instruments	**Roche Diagnostics**
LightCycler® 1.2 and 1.5 Instruments	Discontinued
LightCycler® Software Version 4.10	Cat.-No. 04 779 584 001
LightCycler® Capillaries (20 µl)	Cat.-No. 11 909 339 001
Or	
LightCycler® PRO Instruments	**Roche Diagnostics**
LightCycler® PRO Instrument	Cat.-No. 09 541 713 001
LightCycler® Software Version 1.X.X	Included with Instrument
LightCycler® 480 Multiwell Plate 96 white or	Cat.-No. 04 729 692 001
LightCycler® 480 Multiwell Plate 384 white	Cat.-No. 04 729 749 001
Optional:	

2.3 Instruments

LC Carousel Centrifuge 2.0 (230 Volt)	Cat.-No. 03 709 582 001
Capping Tool	Cat.-No. 03 357 317 001

2.4 Sample Preparation

Manual Sample Preparation:	**Roche Diagnostics**
High Pure PCR Template Preparation Kit	Cat.-No. 11 796 828 001
Nuclease-free PCR grade water	any supplier
Ethanol p.a.	any supplier
Isopropanol p.a.	any supplier
Automatic Sample Preparation:	**Roche Diagnostics**
MagNA Pure Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Discontinued
MagNA Pure 2.0 Instrument	Discontinued
MagNA Pure LC DNA Isolation Kit I	Discontinued
MagNA Pure Compact Instrument	Discontinued
MagNA Pure Compact Nucleic Acid Isolation Kit I	Discontinued
MagNA Pure 96 Instrument	Cat.-No. 06 541 089 001
MagNA Pure 96 DNA and Viral NA Small Volume Kit	Cat.-No. 06 543 588 001
MagNA Pure 96 DNA and Viral NA Large Volume Kit	Cat. No. 06 374 891 001
MagNA Pure 24 Instrument	Cat.-No. 07 290 519 001
MagNA Pure 24 Total NA Isolation Kit	Cat.-No. 07 658 036 001

2.5 Reagents

	Roche Diagnostics
LightCycler FastStart DNA Master HybProbe	Cat.-No. 12 239 272 001

3. Background Information

3.1 Medical Background

Hereditary hemochromatosis is considered to be one of the most common hereditary diseases in population of Caucasian origin.

In European caucasians the prevalence for hemochromatosis is reported to be 1 : 400 (Feder et al., 1996)[1]. About 90 % of the hemochromatosis patients bear a homozygous mutation resulting in amino acid 282 Cys changed to Tyr (282 Y/Y), while another 5 % are compound heterozygote, having the 282 C/Y variation combined with a change from 63 His to Asp for the other allele (63 H/D), or have a homozygous 63 D/D mutation, causing a mild form of hemochromatosis only.

The heterozygote 282 C/Y variant alone shows no risk for hemochromatosis. Heterozygote mutations for 63 H/D or 65 S/C and combined heterozygotes 63/65 without a collateral amino acid change 282 C/Y show no increased risk. 63D and 282Y mutation on the same allele is extremely rare (Best et al., 2001[11]).

The HGVS nomenclature for mutations is related to **p.**rotein or **c.**DNA positions:

p.H63D (rs1799945)	c.187C>G	**63 H/D**	(used here)
p.S65C (rs1800730)	c.193A>T	**65 S/C**	
p.C282Y (rs1800562)	c.845G>A	**282 C/Y**	

Clinical Manifestations

Hereditary hemochromatosis is characterized by an inappropriately high absorption of iron by the gastrointestinal mucosa, resulting in excessive storage of iron particularly in the liver, skin, pancreas, heart, joints and testes.

A well-known manifestion of tissue damage caused by iron accumulation is liver cirrhosis that may lead to hepatocelllular carcinoma (Willis et al., 2000)[2]. Also common for hemochromatosis are arthropathy, hypogonadism, pancreas damage, heart failures, and insulin resistance (Diabetes) (Edwards et al., 1980[3], Pietrangelo, A., 2004[4], Mc Dermott et al., 2005[5], Franchini, M., 2006[6]).

Genetic Testing

In clinical studies it was found that HFE mutations were significantly more frequent in disease than in control specimens (Willis et al., 2000)[2].

The analysis of genes helps us to understand the genetic background for certain diseases; genetic testing alone will identify individuals which might develop a disease but not the disease itself.

Genetic variations can be detected for example by DNA sequencing, hybridization to immobilized probes on arrays or strips, or more convenient by Real-time PCR. Detection of HFE-related mutations by means of a melting curve analysis using fluorescent labeled probes has been published already 1999 (Mangasser et al.1999[7], Bollhalder et al. 1999[8]).

3.2 Methodology and Assay Principle

Using PCR methodology, two fragments of the HFE gene are amplified simultaneously with specific oligonucleotide primers. Fluorescent labeled probes are used to identify the PCR product and to determine the genotype by performing a melting curve analysis.

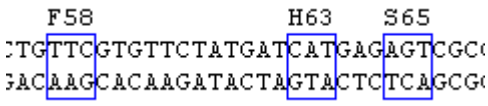
The probe binds to a part of the amplified fragment spanning the mutation site. Any mismatch covered by the probe destabilizes the hybrid. During the melting curve analysis the temperature is slowly increased. The probe dissociates at a specific melting temperature, resulting in a decrease in fluorescence.

Exon 4 : Variant p.C282Y

A 284 bp long PCR fragment containing the c.845G>A (C282Y) polymorphism is analized using a LightCycler® Red 640 red oligomer matching the mutant 282Y allele. In the melting curve analysis the 282Y samples display a higher temperature than the Wildtype C282 allele.
Other variants, eg. 281M or 283P yield different melting temperatures (see 7.6).

Exon 2: Variants p.H63D and p.S65C

A 163 bp long PCR fragment containing the c.187C>G (63 H/D) and c.193A>T (65 S/C) polymorphisms is analized using a SimpleProbe® oligomer which matches the mutant 63D allele.



The probe binds to a part of the amplified fragment starting from amino acid 58 Phe and passing beyond the mutation sites.

In the melting curve analysis, the 63D samples display a higher temperature than the wild type allele 63H, while the 65C samples have the lowest melting temperature. Other variants yield different melting temperatures (see 7.6).

Reading the genotype results is based on the melting temperatures compared to the supplied standards. If permitted by the instrument software reading of the genotype results can be achieved by the automated genotyping (instrument-dependent: software module 'Melt Curve Genotyping').

Automated genotyping results must be reviewed manually for deviating curves and different melting point temperatures. In case that automated typing fails to report consistent genotype results, the genotype must be deducted from the melting temperatures following the criteria described in chapter 7.
See also section 7.6 for melting temperatures expected for other variants.

The kit contains DNA standards encoding for the 282 C/Y, 63 H/D and 65 S/C variants to enable a comparison with clinical samples.

3.3 Performance Characteristics

The test is specific and enables to detect HFE gene variants at codons 63, 65 and 282. No interferences known.

Analytical Specificity

The specificity to the target gene and the suitability of the PCR amplification employed in the present kit was demonstrated by comparison with the results obtained by direct sequencing of the amplicon for *HFE H63D S65C* and the amplicon for HFE C282Y.

Analytical Sensitivity

Detection of serial dilutions of several heterozygous human genomic DNA has revealed that the limit of detection of the present kit is 250 copies DNA (1.5 ng). The smallest amount allowed to be used for testing is 15 ng (see section 1.3.1).

Diagnostic Specificity and Sensitivity

A total number of 120 different genomic DNA samples from individuals of Caucasian origin were analyzed in parallel by sequencing and with the present kit. The study compared results obtained with the kit with ABI 3730xl DNA sequencing data obtained by LGC Genomics GmbH, Berlin.
Study results: Results for both analytical methods were in 100 % concordance.

In particular:

HFE p.H63D S65C: 17 of the samples were heterozygous c.187 C/G (63 H/D) and homozygous wild type c.193 A/A (65 S/S), 93 homozygous wild type 187 C/C and 193 A/A (63 H/H 65 S/S), 4 homozygous wild type 187 C/C (63 H/H) and heterozygous 193 A/T (65 S/C), 6 homozygous mutants 187 G/G (63 D/D) and homozygous wild type 193 A/A (65 S/S); the double homozygous combination mutant 187 G/G and 193 T/T has been never observed.
HFE p.C282Y:118 samples were homozygous wild type 845 G/G (282 C/C), 2 were heterozygous 845 G/A (282 C/Y) while none was homozygous mutant 845 A/A (282 Y/Y).

Summary of results:

93 samples	homozygous 'wild type'	63 H/H	65 S/S	282 C/C
4 samples	heterozygous for 65	63 H/H	65 S/C	282 C/C
15 samples	heterozygous for 63	63 H/D	65 S/S	282 C/C
6 samples	homozygous mutant for 63	63 D/D	65 S/S	282 C/C
2 samples	heterozygous for 63 and for 282	63 H/D	65 S/S	282 C/Y

4. Precautions and Warnings

Handling Requirements

The present product is an *in-vitro* diagnostic device and therefore must be used by qualified personnel only.

General precautions for the handling of generic laboratory materials are required.

The laboratory work-flow must conform to standard practices. Due to the risk of contamination, PCR preparation and PCR amplification must be performed in physically separated areas.

Do not mix reagents from different lots.

Do not use the reagents after the expiration date.

Use the manual version, which is delivered with the kit (see kit label).

Laboratory Procedures

All materials of human origin and related waste must be considered potentially infectious. Thoroughly clean and treat all work surfaces with disinfectants approved by local authorities.

Do not eat, drink or smoke in the laboratory working area.

Do not pipet by mouth.

Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.

Avoid microbial or nuclease contamination of the reagents while pipetting the aliquots. The use of disposable sterile tips with filters is essential.

Thoroughly wash your hands after handling the samples and the sets components.

Sample Preparation

Regarding proper handling and disposal refer to the safety instructions enclosed in the package insert of the product (see chapter 6.1).

Amplification and Detection

Before using this product, please read the LightCycler® Operator's Manual.

Save a sample file to identify each position for correct sample identification.

Check LightCycler® Instrument settings and make sure that they match those reported in the following section "Programming" specific for your Instrument.

Do not touch the capillary surface or plate cover without gloves.

Please refer to all the operative and safety instructions of the LightCycler® Instrument.

Handling of Waste Materials

Dispose of the unused reagents and waste materials according to the current laws.

5. Programming

5.1 Color Compensation

Color Compensation is required for the use of the *LightMix® Kit HFE H63D S65C C282Y.*
Analyze data with 'Color Compensation' (TIB Molbiol CC 530-640).
Its deactivation will generate invalid readouts of the results. For LightCycler® PRO Color Compensation is automatically applied when using the kit specific LightCycler® Analysis Package (LCAP).

5.2 Capillary Based LightCycler® Instruments

For details see the LightCycler® Operator's Manual.
The protocol consists of four program steps (Tab.1):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target °C	95	95	60	72	95	43	75	40
Hold hh:mm:ss	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:20**	**00:00:20**	**00:00:00**	**00:00:30**
Ramp Rate °C/s	**20**	**20**	**20**	**20**	**20**	**20**	**0.2**	**20**
Sec Target °C	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Size °C	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Step Delay Cycles	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Acquisition Mode	None	None	Single	None	None	None	Cont.	None

Tab. 1: Programming of capillary based Instruments.
Note:
While programming maintain default software values: channel = 530, max. samples = 32, seek temperature = 30°C and capillary size = 20 µl. Do not change the capillary size value to 100 µl.
Store the program and the default values as '**RUN Template'** which can be loaded to start every run.

Number of cycles may be increased up to 50 cycles to increase the signals in channel 530.

Just before starting the run, modify max. samples (default = 32) to the number of samples plus controls included in the run to prevent stopping of the instrument due to missing capillaries.

For LightCycler 1.x Instruments using software version 3.5.3 read 'Temperature Transition Rate' instead of Ramp Rate.

5.3 LightCycler® 480 Instruments

For details see the Operator's Manual.

Detection Format: TIB Molbiol 530-640

Please refer to the manual of:
LightMix® Color Compensation HybProbe.
Cat. No. 40-0318-00 , Roche SAP No.: 05997704001

Reaction Volume: 20 µl

Programming:

The protocol consists of four program steps (Tab.2):

1. **Denaturation** of sample and activation of the enzyme
2. **Cycling** PCR-amplification of the target DNA
3. **Melting** Identification of PCR amplified DNA sequence
4. **Cooling** of the Instrument

Step:	**1**	**2**			**3**			**4**
Parameter								
Analysis Mode	**None**	**Quantification mode**			**Melting Curves mode**			**None**
Cycles	1	45			1			1
Target °C	95	95	60	72	95	43	75	40
Acquisition Mode	**None**	**None**	**Single**	**None**	**None**	**None**	**Cont.**	**None**
Hold hh:mm:ss	**00:10:00**	**00:00:05**	**00:00:10**	**00:00:15**	**00:00:30**	**00:02:00**	**00:00:00**	**00:00:30**
Ramp Rate °C/ s **96**	**4.4**	**4.4**	**2.2**	**4.4**	**4.4**	**1.5**	**0.29**	**1.5**
Ramp Rate °C/ s **384**	**4.6**	**4.6**	**2.4**	**4.6**	**4.6**	**2.0**	**0.29**	**2.0**
Acquisitions per °C	**-**	**-**	**-**	**-**	**-**	**-**	**1**	**-**
Sec Target °C	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Size °C	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**
Step Delay cycles	**0**	**0**	**0**	**0**	**-**	**-**	**-**	**-**

Tab. 2: Programming of LightCycler® 480 Instruments family

Note:

a) Store the program and the default values as '**RUN Template'**, which can be loaded to start every HFE LightCycler® run.
b) Ensure to program only 1 acquisition per second instead the default value 3; more acquisitions reduce the slope of the melting curve, increase experiment time and cause kit malfunction.
c) Number of cycles may be increased up to 50 cycles to increase the signals in channel 530.

5.4 LightCycler® PRO Instrument

LightCycler® PRO Instrument

Use the software version 1.X.X. See the LightCycler® PRO System User Assistance for details.

For a matching LightCycler® Analysis Package (LCAP) file for LightMix® *HFE 63-65-282*, please visit navifyportal.roche.com to download. Please check for the latest version of the LCAP.

The kit-specific run profile is part of the LCAP and equivalent to the run conditions shown above (Tab.2).

LightCycler® Analysis Package:	1013_HFE_96
Detection Format:	FAM (494/523), FAM + LC Red 640 (494/636)

Save the LCAP file in the assay folder of the SFTP or USB device.

Import and install the downloaded LCAP onto the LightCycler® PRO Instrument and activate it.

Create or import a plate setup in the Plates tab.

6. Experimental Protocol

Program the Instrument before preparing the solutions (see 5. Programming and read the Operator's Manual for details).
The described performance of the assay can be guaranteed only when used with Roche Diagnostics LightCycler® systems or cobas z 480 Analyzer.

6.1 Sample Preparation

For preparation of genomic DNA use human peripheral blood (EDTA, citrate). The use of heparin is strongly discouraged since this anticoagulant might interfere with the PCR.
Perform nucleic acid purification using the High Pure PCR Template Preparation Kit or the MagNA Pure Instruments with the extraction kits appropriate to the MagNA Pure Instrument used (see 2. Additional Devices and Reagents) as described in the respective protocols.

6.2 Reagents Preparation

6.2.1 Preparation of Parameter-Specific Reagents (PSR)

►	Each **PSR** tube is sufficient for 32 reactions.
1	Spin the premixed **PSR** tube at 10,000 RPM for 1 minute.
2	Check that the pellet is located at the bottom.
3	Add **66 µl** of PCR-grade **Water** to the **PSR** tube.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use 2 µl of **PSR** for a 20 µl PCR reaction.

6.2.2 Preparation of Standards

►	Each **Standard** reagent tube is sufficient for 32 reactions.
1	Spin the three tubes at 10,000 RPM for 1 minute.
2	Check that the blue pellet is located at the bottom of the tube.
3	Dissolve pellet by adding 160 µl PCR-grade **Water**.
4	Incubate for 20 sec at room temperature.
5	Vortex for 10 sec.
6	Spin the tubes to collect drops.

► Use **5 µl** of each **Standard** for a 20 µl PCR reaction.
► All three **Standards** must be used in each run. For Light Cycler® PRO also the combination of the three Standards is mandatory.
Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.2.3 Preparation of Genotyping Standards

The LightCycler® software 4.05 and later (capillary based instruments) and software 1.5 and later (LightCycler® 480 instruments) and software 1.X.X (LightCycler® PRO) can be calibrated with reference standards to perform an automated genotyping of unknown clinical samples

►	Genotyping Standards must be generated by mixing the Standards as follows:
	Prepare 3 clean tubes and label them as 5, 6 and 7
Tube 5	Mix 5 µl of **Standard 1** with 5 µl of **Standard 3**
	Genotype for p.C282Y and p.S65C
Tube 6	Mix 5 µl of **Standard 1** with 5 µl of **Standard 2**
	Genotype for p.C282Y and p.H63D
Tube 7	Mix 5 µl of **Standard 2** with 5 µl of **Standard 3**
	Genotype for 65C / 63D

► Use **5 µl** of Genotyping Standards (Tube 5-7) for a 20 µl PCR reaction

► All three Genotyping Standards must be used in the first run of the kit to calibrate the genotyping module. For Light Cycler® PRO the combination of the three Genotyping Standards is mandatory for each run

Please note: Opening the vials may cause contaminations of the work-space (aerosol).

6.3 Preparation of the Reaction Mix

6.3.1 Preparation of 32 LightCycler® Reaction Mix

We recommend preparing 32 reactions to prevent storage of dissolved or activated reagents in varying volumes (6.2). See chapter 6.4 for storage and stability of dissolved components. For preparation of the reaction mix for less samples, please go to step 6.3.2 “Reaction mix for single reaction”.

Prepare the reaction mix in the PSR tube (cooled):

Components	**32 reactions**
To the **PSR** tube (red cap)	66.0 µl
Add:	
H_2O, PCR-grade (colorless cap)	310.2 µl
$MgCl_2$ solution 25 mM (blue cap)	52.8 µl
LightCycler® FastStart DNA Master HybProbe (red cap)	66.0 µl
⚠ Substitute of the “long neck cap” of the **PSR** tube with the red cap from FastStart	
Total Volume	**495.0 µl**

Tab. 3: Volumes of components for preparing 32 reaction mixture

6.3.2 Preparation of the Single LightCycler® Reaction Mix

Prepare the reaction mix by multiplying each volume (Tab. 4) by the number of biological samples to be analyzed plus five reactions (**NTC**, three **Standards**, one excess) and (optionally) three **Genotyping Standards**. For LightCycler® PRO **all Standards, Genotyping Standards** and **NTC** are mandatory for **each** run.

Prepare the reaction mix in a cooled vial:

Components	**Single reaction**
H_2O, PCR-grade (colorless cap)	9.4 µl
$MgCl_2$ solution 25 mM (blue cap)	1.6 µl
PSR (red cap), see **6.2.1**	2.0 µl
LightCycler® FastStart DNA Master HybProbe (red cap)	2.0 µl
Volume of reaction mix	**15.0 µl**

Tab. 4: Volumes of components for preparing a single reaction mixture

Gently pipette up and down the reaction mix.
A high percentage of experimental failure is due to a non homogeneous reaction mix!

6.3.3 Capillary / Well Loading Procedure

Each run must include one **NTC** to demonstrate the absence of contaminations with genomic DNA or HFE PCR product and three Standards to identify run specific melting temperatures. Regulatory agencies or local laboratory rules might require including also the three Genotyping Standards.

Capillary / Well Loading Procedure

▶	Remember to include the controls when setting up the run.
1	Mix gently, spin down and check for the absence of air bubbles in the reaction mix vial.
2	Dispense **15 µl** per capillary/well of reaction mix
3	**Mandatory:** Add **5 µl** of PCR-grade **H_2O** as **NTC** in position 1 (A1). Add **5 µl** of **Standard 1** in position 2 (A2). Add **5 µl** of **Standard 2** in position 3 (A3). Add **5 µl** of **Standard 3** in position 4 (A4). **Optional but mandatory for LightCycler® PRO:** Add **5 µl** of **Tube 5** Genotyping Standard in position 5 (A5). Add **5 µl** of **Tube 6** Genotyping Standard in position 6 (A6). Add **5 µl** of **Tube 7** Genotyping Standard in position 7 (A7).
4	Add **5 µl** of **Sample** in the remaining capillaries / wells.
5 ⚠	Close the capillary/plate and centrifuge. Check that no air bubbles are present.
6	Place the rotor/plate into the Instrument.
7	Capillary-based users only: input number of samples.
8	Start the run.
9	Input experiment's name when instructed.
10	Save sample data in the samples' window.

6.4 Storage and Stability of Dissolved Components

Reaction Mix
The complete reaction mix containing Parameter-Specific Reagents (**PSR**), LightCycler® FastStart DNA Master HybProbe and $MgCl_2$ can be stored refrigerated (2 °C – 8 °C) for up to 30 days.

Avoid prolonged exposure to light.

Parameter-Specific Reagents (PSR)
The dissolved PSR is stable for up to 30 days when stored refrigerated (2 °C – 8 °C).

Avoid prolonged exposure to light.

Positive Controls and Genotyping Standards
The dissolved Positive Control and Genotyping Standards are stable for up to 30 days when stored refrigerated (2 °C - 8°C).

6.5 Loading of Controls and Genotyping Standards

Samples in positions 1 to 4 must be filled in each run as described in the table below. Samples in positions 5 to 7 are required for teaching of Genotyping Standards only in the first run of the kit.

Genotype results are based on melting temperatures.The use of the automated genotyping module present in the LightCycler® 2.0 and LightCycler® 480 software is optional but is mandatory for LightCycler® PRO.

Refer to LightCycler® Operator's Manual for details.

6.5.1 Capillary Based Instruments

In "Samples data - Capillary View", input Sample Name as described in the second column. Select "Analysis Type – Genotyping". Select Channel 530 and 640 only! From the pull down menu select "Sample Type" and copy the "Genotype" description.

Pos	Sample Name	Channel	Target Name	Sample Type	Genotype (Amino Acids)
1	**NTC**	530	Target 1	NTC	
		640	Target 4	NTC	
2	**Standard 1**	530	Target 1	Melting Standard	HFE 63H 65S Wild Type
		640	Target 4	Melting Standard	HFE 282Y Mutant
3	**Standard 2**	530	Target 1	Melting Standard	HFE 63D Mutant
		640	Target 4	Melting Standard	HFE 282C Wild Type
4	**Standard 3**	530	Target 1	Melting Standard	HFE 65C Mutant
		640	Target 4	Unknown	
5	**Tube 5**	530	Target 1	Melting Standard	HFE 65 S/C Heterozygous
		640	Target 4	Melting Standard	HFE 282 C/Y Heterozygous
6	**Tube 6**	530	Target 1	Melting Standard	HFE 63 H/D Heterozygous
		640	Target 4	Unknown	
7	**Tube 7**	530	Target 1	Melting Standard	HFE 65C / 63D Heterozygous
		640	Target 4	Unknown	

6.5.2 LightCycler® 480 Instruments

In the “Sample Editor” window, in “Step1: Select Workflow” section, select “Melt Geno”. Input the description of **Standards** and **Genotyping Standards** as follows:

Pos	Filter combination	Sample Name	Melt Geno Sample Type	Melt Geno Genotype
1	Fluos (465-510)	**NTC**	NTC	
	Red 640 (498-640)	**NTC**	NTC	
2	Fluos (465-510)	**Standard 1**	Melting Standard	HFE 63H 65S Wild Type
2	Red 640 (498-640)	**Standard 1**	Melting Standard	HFE 282Y Mutant
3	Fluos (465-510)	**Standard 2**	Melting Standard	HFE 63D Mutant
3	Red 640 (498-640)	**Standard 2**	Melting Standard	HFE 282C Wild Type
4	Fluos (465-510)	**Standard 3**	Melting Standard	HFE 65C Mutant
4	Red 640 (498-640)	**Standard 3**	Unknown	
5	Fluos (465-510)	**Tube 5**	Melting Standard	HFE 65 S/C Heterozygous
5	Red 640 (498-640)	**Tube 5**	Melting Standard	HFE 282 C/Y Heterozygous
6	Fluos (465-510)	**Tube 6**	Melting Standard	HFE 63 H/D Heterozygous
6	Red 640 (498-640)	**Tube 6**	Unknown	
7	Fluos (465-510)	**Tube 7**	Melting Standard	HFE 65C / 63D Heterozygous
7	Red 640 (498-640)	**Tube 7**	Unknown	

For the cobas z 480 Analyzer use channel 498-645 instead of 498-640.

6.5.3 LightCycler® PRO Instruments

In the plate setup the matching LCAP has to be selected. Melting Standards and NTC have to be assigned to the associated well position.

Sample ID	Sample Role	Channel	Genotype/ Targetname
1013_HFE_NTC	NTC	FAM	n.a.
		LC Red640	
Standard 1	Melting Standard	FAM	H63 S65 / H63 S65
		LC Red640	282Y / 282Y
Standard 2	Melting Standard	FAM	63D S65 / 63D S65
		LC Red640	C282 / C282
Standard 3	Melting Standard	FAM	H63 65C / H63 65C
		LC Red640	-
Standard 1+2	Melting Standard	FAM	H63 S65 / 63D S65
		LC Red640	C282 / 282Y
Standard 1+3	Melting Standard	FAM	H63 S65 / H63 65C
		LC Red640	-
Standard 2+3	Melting Standard	FAM	63D S65 / H63 65C
		LC Red640	-

Run Profile Name: Simple Probe 530 - 640

7. Data Analysis and Interpretation

7.1 Calibration

Calibrate as described in sections 6.3.3, 6.5, 7.2.2 and 7.2.3.

7.2 Quality Control – Acceptance Criteria

In order to perform a reliable genotyping analysis, it is essential that **NTC** and all three **Standards** are included in each run. For LightCycler® PRO in addition also all three Genotyping Standards are mandatory.

NOTE: The test is performed at an annealing temperature of 60ºC at which the probes will not bind well, yielding low or even no signals in 'quantification'. For this reason, the acceptance criteria are based only on the definition of the melting-curve patterns as described below.

7.2.1 No Template Control

NTC (Mandatory - Position 1).
Melting-curve analysis of the NTC must provide a negative result: no assay-specific melting peaks (see 7.2.2) must be detected.
In case that the **NTC** should report one or more specific peaks a contamination or a pipetting error has occurred; the session is not valid and the procedure has to be repeated. If the problem sustains, change water and/or reagents and repeat.

In this case - to enable the automatic genotyping – change the NTC sample from “No Template Control” to “Unknown” (see paragraph 6.5); alternatively, results must be read from the melting temperatures (see paragraph 7.5). Not applicable with LightCycler® PRO Instruments.

7.2.2 Standards (Positive Controls)

Melting-curve analysis should always show
HFE **Standard 1** (Mandatory - position 2).
one melting peak in channel 530
one melting peak in channel 640
HFE **Standard 2** (Mandatory - position 3).
one melting peak in channel 530
one melting peak in channel 640
HFE **Standard 3** (Mandatory - position 4).
one melting peak in channel 530
one melting peak in channel 640
The provided **Standards** mimic **homozygous** clinical samples.
See 7.5 Interpretation of the Results for expected melting temperatures.

7.2.3 Genotyping Standard

Mandatory when using with LightCycler® PRO. Melting-curve analysis should always show:
Tube 5 Genotyping Standard (Optional - position 5).
two melting peaks in channel 530
two melting peaks in channel 640
Tube 6 Genotyping Standard (Optional - position 6).
two melting peaks in channel 530
two melting peaks in channel 640
Tube 7 Genotyping Standard (Optional - position 7).
two melting peaks in channel 530
one melting peak in channel 640
Genotyping Standards mimic **heterozygous** clinical samples.

See **7.5 Interpretation of the Results** for expected melting temperatures.

Before repeating a run consider common errors; check in particular the amplification profile, correct master-mix and $MgCl_2$ concentration used, and keep in mind that also inadequate storage of reagents may cause a failure of the device.

7.2.4 Samples

The result of the assay must always show one or two melting peaks for each channel. See 7.5 Interpretation of Results **expected melting temperatures.**

⚠ No more than two peaks per sample are expected in channel 530.
The melting peak profiles must be conformable to the acceptance criteria described in the present chapter and in **7.5 Interpretation of the Results** See also **7.5.1 Problematic profiles** and **7.6 Rare Variants**.

7.2.5 Abnormal Melting Curves

An unexpected melting curve might be due to an incorrect sample preparation, a defect in the product, or a variant under the probe binding region. The whole procedure has to be repeated (sample preparation, amplification, and detection). If an abnormal melting curve persists, another method must be used for the identification of the sequence.
For the purpose of product improvement and post-market surveillance, please send deviant melting samples to TIB Molbiol GmbH, Berlin laboratories.
Contact service@tib-molbiol.de before sending. Examples of known variants are depicted in paragraph 7.7. Rare Variants.

7.3 Saving External Genotyping Standards

(Not applicable for LC1.x software versions below 4.0, LightCycler® 96 and for LightCycler® PRO Instruments).

After the genotyping analysis, if samples 1 to 7 comply with the acceptance criteria (see **7.3 Quality Control – Acceptance Criteria**), save the Genotyping Standards as follows and use External Standard in all successive runs.

7.3.1 Capillary Based Instruments

In the Melting Curve analysis - Genotyping window open the “Standard (Int)” menu and select “Save standards as External”.

7.3.2 LightCycler® 480 Instruments

In the Melt Curve Genotyping analysis window, open the “Standards (In-run)” menu and select “Save as ext.”

7.3.3 LightCycler® PRO Instruments

Genotyping Standards have to be used in **each** run.

7.4 Reading the Results

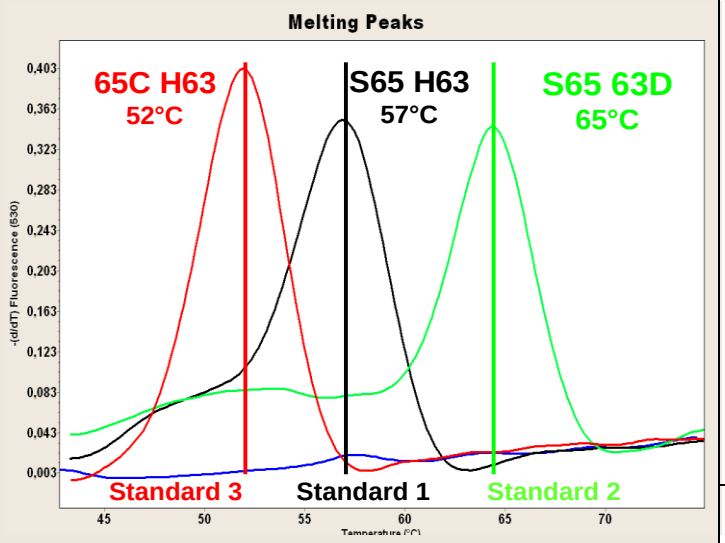
Perform data analysis as described in the Instrument Operator's Manuals.

7.4.1 Melting Analysis: Capillary Based Instruments

The melting-curve peaks (Fig. 1 and 2) display homozygous genotypes.

⚠Activate the Color Compensation to avoid wrong results.

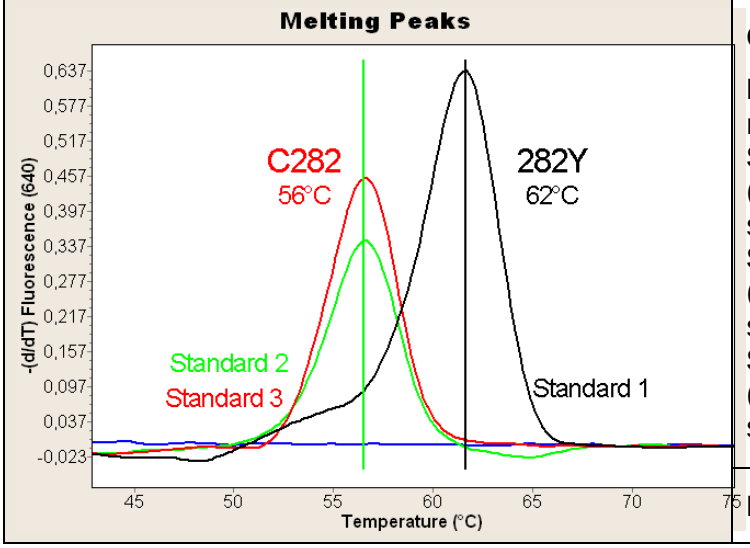
View *HFE H63D S65C C282Y* data for Melting as follows:
LC 2.0 Instrument (or LC1.x with software versions 4.1):
View Melting data for HFE H63D S65C in channel 530 (Fig. 1) and C282Y in channel 640 (Fig. 2).
Analysis Type "Melting Curve Analysis – Genotyping" mode.



Channel 530

NTC (blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 57 °C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 65 °C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 52 °C.

Fig. 1: Sample data HFE H63D S65C.



Channel 640

NTC (blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 62 °C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 56 °C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 56 °C.

Fig. 2: Sample data HFE C282Y.

Note: The values of the melting temperatures may vary ±2.5 °C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5 °C.
In case of variation, see instructions: **7.3.5 Abnormal Melting Curves** and **7.7.3 Rare Variants**.
In case of low signals in channel 530 the number of cycles can be increased up to 50 cycles.

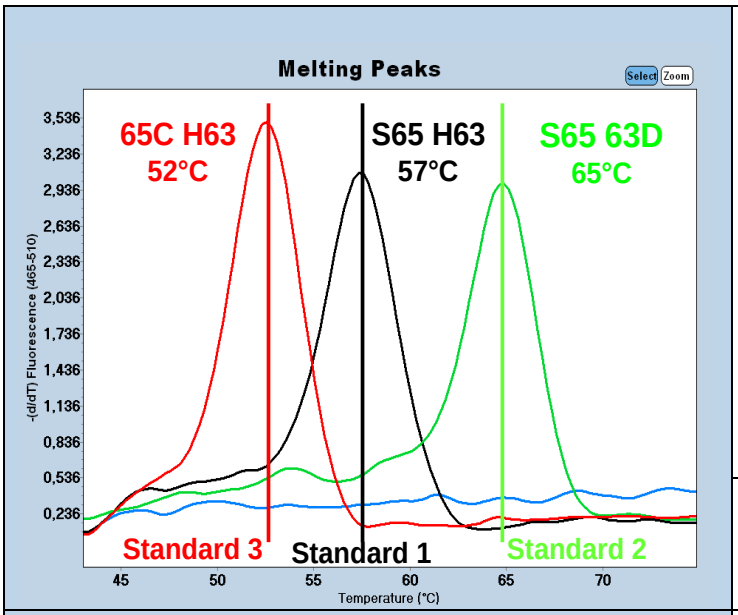
In case of automatic genotype module failure (score <0.6 or res <0.4), switch to manual reading (Tm calling) and compare with table 5.1&5.2 (7.5. Interpretation of the Results).

7.4.2 Melting Analysis: LightCycler® 480 Instruments

The melting-curve peaks (Fig. 3 and 4) display homozygous genotypes.

⚠ Activate the Color Compensation to avoid wrong results.

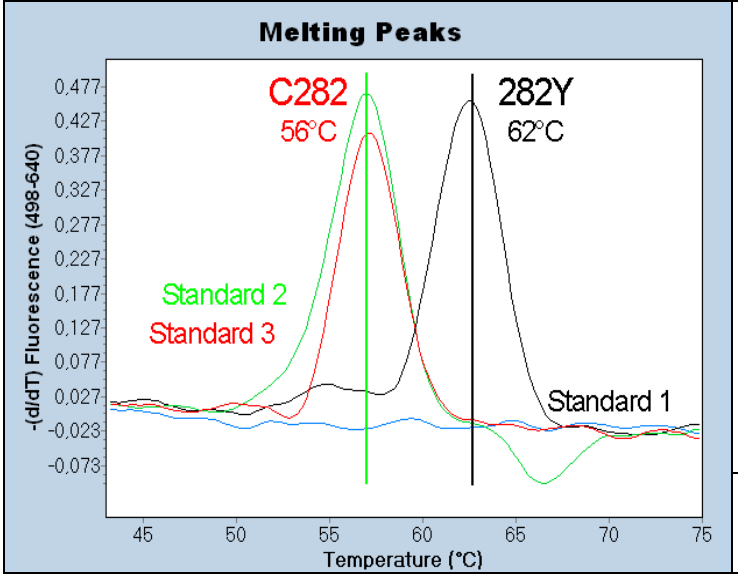
View data for Melting as follows:
For use in LightCycler® 480 Instrument, view HFE H63D S65C Melting data in channel 483-533 and HFE C282Y in channel 483-640, “Melt Curve Genotyping” mode.
For use in LightCycler® 480 II Instrument, view HFE H63D S65C Melting data in channel 465-510 (Figure 3) and HFE C282Y in channel 498-640 (Figure 4) “Melt Curve Genotyping” mode.
For use in cobas z 480 Analyzer, view HFE H63D S65C Melting data in channel 465-510 and HFE C282Y in channel 498-645. Analysis Type “Melt Curve Genotyping” mode.



Channel 465-510

NTC (blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 57 °C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 65 °C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 52 °C.

Fig. 3: Melting sample data for HFE H63D S65C.



Channel 498 - 640

NTC (blue line–position 1)
must show a flat line (no melting peak).
Standard 1 Positive Control
(black line – position 2)
shows a melting peak at 62 °C.
Standard 2 Positive Control
(green line– position 3)
shows a melting peak at 56 °C.
Standard 3 Positive Control
(red line – position 4)
shows a melting peak at 56 °C.

Fig. 4: Melting sample data for HFE C282Y.

Note: The values of the melting temperatures may vary ±2.5°C between different experiments.
The ΔT between the melting peaks for heterozygous genotypes may vary ±1.5°C.
In case of variation, see instructions: **7.6 Rare Variants**.
In case of low signals in channel 530 the number of cycles can be increased up to 50 cycles.

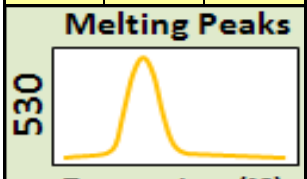
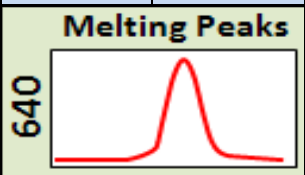
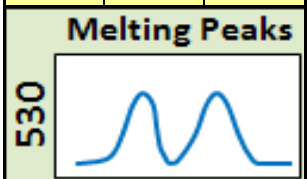
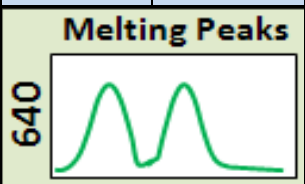
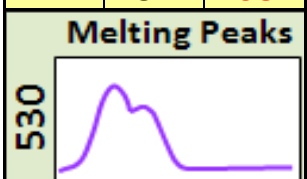
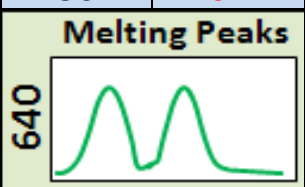
In case of automatic genotype module failure (score <0.6 or res <0.4), switch to manual identification of melting curve (Tm calling) and compare results with table 5.1&5.2 (7.5. Interpretation of the Results).

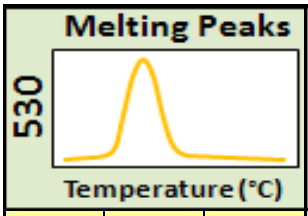
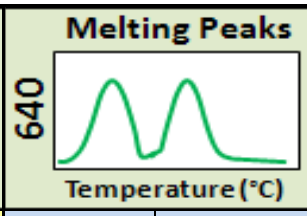
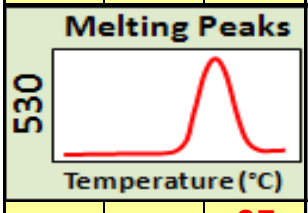
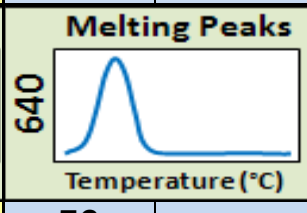
7.4.3 Melting Analysis: LightCycler® PRO Instruments

Perform data analysis as described in the LightCycler® PRO System User Assistance. Review and approve the results in the Target Results tab. It is necessary to approve the results of **Standards**, **Genotyping Standards** and **NTC** before approving the results of unknown samples. It is possible to overwrite results, overwritten results will be flagged. In the Sample Results tab approved results can be released. In case of low signals in channel FAM the number of cycles can not be increased.

7.5. Interpretation of the Results

1) Start to read results in channel 640 and identify all **high risk** individuals having the homozygous **282 Y/Y** mutation (single high-melting peak) [1].
   ***Note**: Homozygous 282Y/Y samples must be 63 His wild type; see channel 530).*
2) Still in channel 640 select all **282 H/Y** heterozygous individuals and analyze them in channel 530 to identify **63H/D** individuals having a **low risk** [2].
   Report remaining 282 H/Y heterozygous which are either **63 H/H** and **65 S/S** or **65S/C** as '**no risk**' (general population risk) [3,4].
   ***Note**: 282 H/Y heterozygous samples can not be 63 D/D nor 65 C/C nor 63 D/65 C.*
3) Read channel 530 to select all individuals with the single high-melting peak which are homozygous **63 D/D** (**mild form**) [5]
   ***Note**: Homozygous 63 D/D samples are commonly wild type in channel 640. See ref. 11.*

Channel 530			Channel 640		Common Name	Disease
63H 65C	63H 65S	63D 65S	C282	282Y	HFE Genotype	Risk
					**282Y homozygote** **H63 S65 | H63 S65** **282Y | 282Y**	**[1]** **High risk**
-	57	-	-	62		
					**H63D heterozygote** **C282Yheterozygote** Compound heterozygote **H63 S65 | 63D S65** **C282 | 282Y**	**[2]** **Low risk**
-	57	65	56	62		
					**S65C heterozygote** **C282Yheterozygote** Compound heterozygote **H63 S65 | H63 65C** **C282 | 282Y**	**[3]** **Putative increased risk** **(disputed)**
52	57	-	56	62		

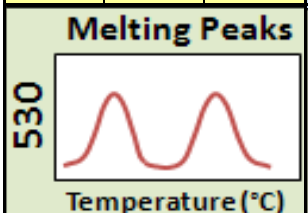
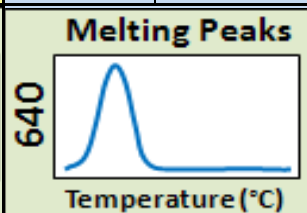
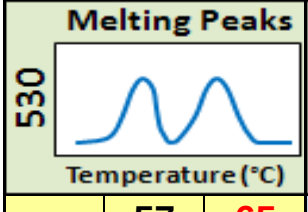
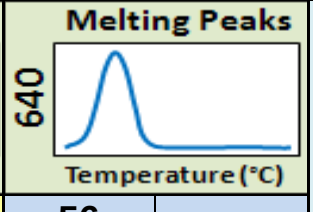
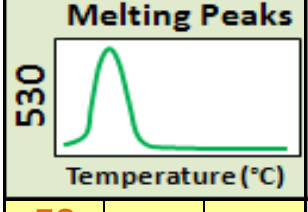
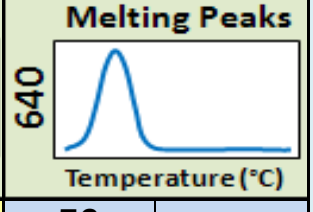
					**C282Y heterozygote** **H63 S65 | H63 S65** **C282 | 282Y**	**[4]** **General Population Risk**
-	57	-	56	62		
					**63D homozygote** **63D S65 | 63D S65** **C282 | C282**	**[5]** **Mild Form**
-	-	65	56	-		
ΔTm 5 °C	ΔTm 8 °C		ΔTm 6°C		**Tab. 5.1: HFE 282 and disease risk variants**	
ΔTm 13°C					See also 7.3.4 and 7.3.5	

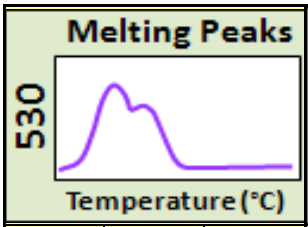
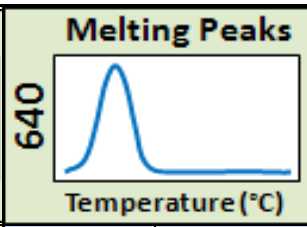
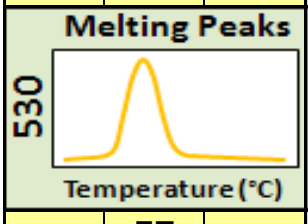
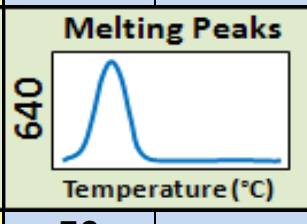
4) Report the status of all remaining **282 C/C (wild type)** patients for their melting profile channel 530 as **'no risk'** (General population risk) [6-10].

Note: 65 S/C heterozygous may have only a shoulder instead of a separated peak at the temperature for 63 H. The shape of the curves for all variants is significant if inspected by eye since it may be called wrong by the instrument software, irrespective if using the 'Genotyping' module or the 'Tm Calling' program.

Note: The values of the melting temperatures (Tm) may vary ±2.5 °C between runs. The ΔT for melting peaks for heterozygous genotypes may vary ±1.5 °C

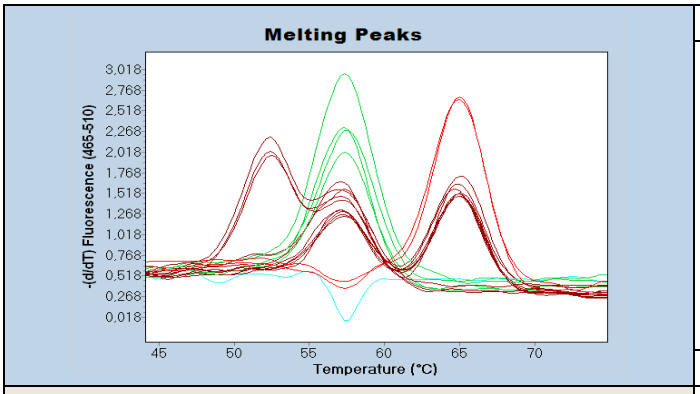
In case that the peaks in channel 530 are low consider to run up to 50 cycles.

Channel 530			**Channel 640**		**Common Name**	**Disease**
63H 65C	**63H 65S**	**63D 65S**	**C282**	**282Y**	**HFE Genotype**	**Risk**
					**65C / 63D heterozygote** Compound heterozygote **H63 65C | 63D S65** **C282 | C282**	**[6]** **General Population Risk**
52	-	65	56	-		
					**H63D heterozygote** **H63 S65 | 63D S65** **C282 | C282**	**[7]** **General Population Risk**
-	57	65	56	-		
					**65C homozygote** **H63 65C | H63 65C** **C282 | C282**	**[8]** **General Population Risk**
52	-	-	56	-		

					**S65C heterozygote** **H63 S65 | H63 65C** **C282 | C282**	**[9]** **General Population Risk**
52	**57**	**-**	**56**	**-**		
					**Wild Type** **H63 S65 | H63 S65** **C282 | C282**	**[10]** **General Population Risk**
-	**57**	**-**	**56**	**-**		
ΔTm 5°C	ΔTm 8°C		ΔTm 6°C		**Tab. 5.2: C282 with common 63/65 variants**	
ΔTm 13°C					See also 7.3.4 and 7.3.5	

7.5.1 Interpretation of Problematic Profiles

In the figures below, melting peak profiles from clinical samples are depicted.

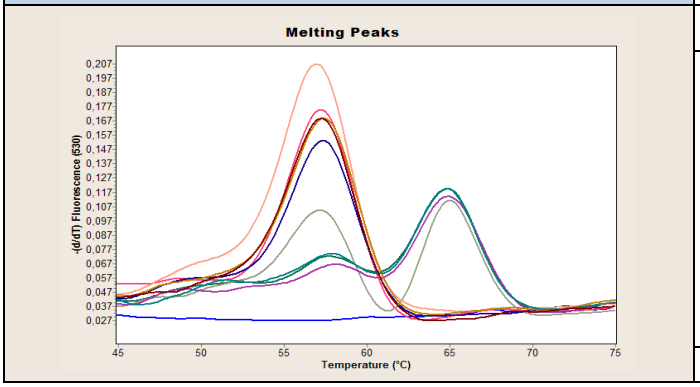


Instrument: LC480 II

Channel 465 - 510

NTC (light blue line): the no template control might show an abnormal negative peak, without affecting the automatic identification of other samples.

Fig. 5 Melting peaks HFE H63D S65C.



Instrument: LC2.0

Channel 530

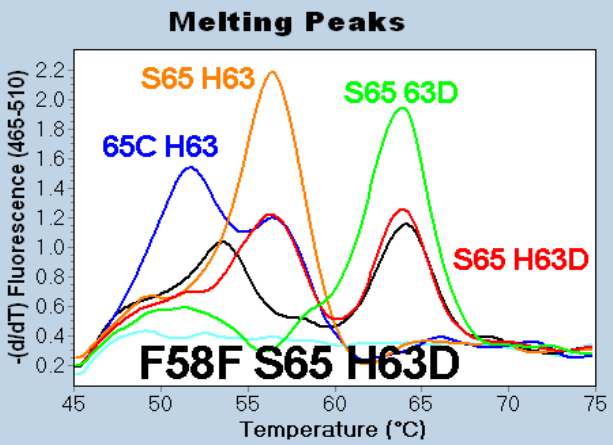
The shape of the curve can be influenced by the allele condition in the other channel without interfering with correct interpretation.

The green or violet and gray curves display two peaks (57 °C and 65 °C) and, despite the different shape of the curve, must be interpreted as heterozygous

Fig. 6 Melting peaks HFE H63D S65C.

7.6 Rare Variants

The sequences used in this device do not interfere with other known gene variants; new variants will usually generate a different Tm peak than WT or MT. To demonstrate the ability of the assay to discriminate the correct genotype, synthetic targets are used to mimic all the variants reported in GeneBank (November,2023). The absolute Tm values obtained with synthetic targets might differ from the ones resulting from biological samples, while the **relative ΔTm must remain constant**.
The present kit is not intended to identify variants other than specified in section **1.3 Specifications**. Another method must be used for the identification of sequences presenting abnormal melting peaks.



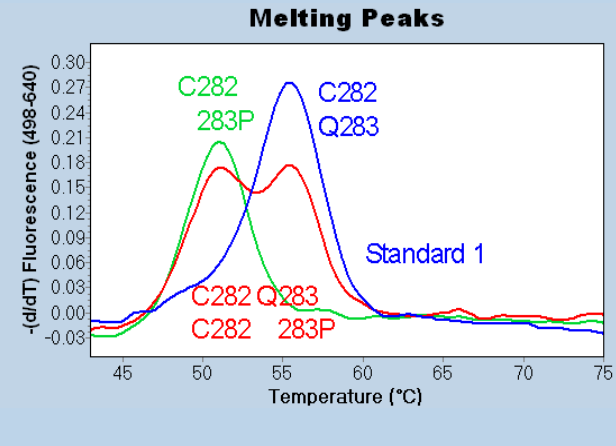
Instrument: LC480II

Exon 2: Channel 465 - 510

The silent mutation g.8658C>T p.**F58F** (rs147297176) has been observed in 2012 for one patient with a melting peak at **54 °C** - which is between S65 H63 (wild type) and 65C H63.
The patient was g.8658C>T heterozygous (black line, left peak) combined with 63D. [10]
At time of observation (2013) this polymorphism was not registered in dbSNP at NCBI.

No reference standard provided with the kit.

Fig. 7 Melting peaks for HFE F58F.



Instrument: LC480II

Exon 4: Channel 498 - 640

Variant p.**T281M** (rs915273578) generates a Tm at **47 °C** (not shown), published Biasiotto et al, 2008[12]
Variant p.**Q283P** (rs111033563) generates a Tm at **51 °C** (green), published by Ka et al. 2005 [9]
282C 283Q / 282C 283Q (wild type, blue) 56 °C
282C 283Q / 282C 283P (red) 56 °C / 51 °C.
282C 283P / 282C 283P (green) 51 °C

No reference standards provided with the kit.

Fig.8: Melting peaks for HFE Q283P.

8. Troubleshooting

Instrument	Capillary based instruments	LightCycler® 480 and PRO instruments
Event	**Possible Reason**	**Solution**
No sample detected	No centrifugation	Centrifuge capillary
All PCRs	Incorrect selection of detection channel	Set correct channel before analyzing
	Incorrect amplification protocol	Check instrument program
Low 530 signals	The HFE 63/65 fragment is known to have a lower PCR efficiency	Run more PCR cycles. Up to 50 cycles have been evaluated / yield same results
Inconsistent baseline among various samples	Incorrect pipetting	Ensure homogeneity of mix quantity in each sample
	Non homogenous reaction mix	Pipette reaction mix up and down 10 x with a clean 200µl tip before distribution in reaction vessel
	Multiwell plate badly sealed	Ensure that multiwell plate is properly sealed
Baseline "Saw teeth like"	Bubble in well	Centrifuge plate before run
	Incorrect positioning of capillary in the carousel	Firmly press capillary in the carousel
No signal in the Positive Control	Error while setting the instrument	Check the position settings of the Positive Control
	Incorrect PSR / $MgCl_2$ concentration	Repeat assay
	Positive Control or standard degradation	Use a new aliquot of Positive Control or standard
Positive signal in No Template Control **NTC**	Error while setting the instrument	Check the position settings of the NTC
	Dispensing error	Comply with the work sheet when dispensing samples, NTCs, Positive Controls and standards
	Dispensing error	Always change tips among samples
	Dispensing error	Avoid spilling the contents of the sample test tube
	Contamination of the PCR- grade water.	Use a new aliquot of PCR- grade water
	Contamination of the reaction mix	Use new aliquots of reagents to prepare the reaction mix
	Contamination of the extraction/preparation area for amplification reactions	Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use
	Contamination of the extraction/preparation area for amplification reactions	Add LightCycler® Uracil-DNA Glycosylase (Cat.-No.03 539 806 001) to the reaction mix according to instructions
No signal in samples	Low DNA amount	Check DNA concentration
	Sample inhibition	Dilute sample and repeat PCR, or repeat extraction and PCR, or add Positive Control and repeat
Melting curve outside the expected temperature range	With peaks Tm **concordant** with Positive Control: Incorrect reagent concentration	Manually assign results accordingly to Positive Control
	With peaks Tm **discordant** with Positive Control: Possible extraction inhibitor	Repeat assay diluting the DNA 1:3
	With peaks Tm **discordant** with Positive Control: Possible different mutation	Repeat assay by sequencing and report unexpected variant to service@tib-molbiol.de

9. References

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Version History

Notes in red mark events that require to change laboratory procedures
Notes in blue mark improvements or change in composition

V160626	Storage (1.1, 1.4). Editorial changes.	26-06-2016
V170303	Editorial. rs915273578 (=T281M) listed (1.3); Misleading wording corrected (7.3.1); update reference figure labelling (7.5.1 and 7.5.2). Storage conditions clarified (1.1, 1.4, 6.4)	11-10-2017
V241024	Addition of the LightCycler® PRO and MagNa Pure24. LightCycler® Nano and FastStart DNA Master HybProbe removed. Editorial changes.	24-10-2024

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